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Circulating Tumour Cells in locally advanced head and neck cancer: Preliminary report about their possible role in predicting response to non-surgical treatment and survival

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Abstract ***Background and purpose:*** The mechanism of dissemination of locally advanced head and neck cancer (LAHNC) is far to be resolved. Circulating Tumour Cells (CTC) have been identified as a prognostic factor in metastatic breast and prostate cancer. This prospective multi-centric analysis studied the possible role of CTC identification in LAHNC.

Materials and methods: CTC were searched in 73 patients with LAHNC (oropharynx, $n = 39$; nasopharynx, $n = 10$; larynx, $n = 10$; paranasal sinuses, $n = 6$, of whom 3 with sinonasal undifferentiated carcinoma, SNUC; hypopharynx, $n = 5$; oral cavity, $n = 3$). All of them (apart from SNUC) had squamous cell cancers. The relationship between CTC positivity and other clinical prognostic factors has been investigated. Response to treatment and survival has been related with changes in CTC number during the treatment.

Results: CTC were frequently identified in oro- and hypopharyngeal cancer and in SNUC. They were more frequent in stage IV than in stages I–III disease (18% versus 6%, $p = $ NS (not significant)). Partial or complete response (CR) was related with the absence or disappearance of CTC during treatment ($p = 0.017$). A decrease in the CTC number or their absence throughout the treatment seems also related with non-progressive disease, after both complete or incomplete remission and with the proportion of patients alive and NED (no evidence of disease) ($p = 0.009$).

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Conclusions: These preliminary data suggest a possible role of CTC determination in head and neck cancer. Additional and longer follow up data need to be collected to confirm these findings.

1. Introduction

Cancer arises as organ-confined, but eventually spreads to distant sites through the bloodstream, generating metastases that are often the cause of death.

The process by which a tumour cell escapes from the primary site, survives in the blood flow and then colonises distant organs (metastases)[1] or again the primary site of disease (*tumor self-seeding*),[2] is not very well known. '*Epithelial to mesenchimal transition*' (EMT), the process through which epithelial cells acquire a mesenchimal identity, could be recognised as an essential feature of tumour invasion. By this process, tumour cells could acquire the capacity to detach from the primary mass, to enter blood or lymph vessels, to exit the vessels and finally to re-grow in the distant sites.[3] The early spread of tumour cells is usually not detected even by high-resolution imaging technologies, preventing potentially effective early detection.

Because Circulating Tumour Cells (CTC) probably encompass features of *differentiated* and *stem-like tumor cells* and recapitulate the heterogeneous composition of the primary lesion,[4] their detection in peripheral blood could guide to the identification of properties consistent with EMT phenotype or explain the resistance of CTC to chemo and radiotherapy. The most promising application of CTC detection lies in their possible use as a non-invasive approach for tumour cell genotyping; this can be repeated during the treatment, to monitor the acquisition of novel genetic abnormalities in response to chemo-radiotherapy (*chemo-radio resistant phenotype*). This general scenario holds true also for head and neck cancer, increasingly treated nowadays with radiotherapy and chemotherapy in the context of organ and function sparing strategies.

CTC are very rare in the circulation and their identification is highly dependent from the approaches used for their isolation, the most successful being those based the cell adhesion protein epithelial cell adhesion molecule (EpCAM), commonly expressed by *epithelial cells* but absent in normal blood cells. Immuno-magnetic capture methods consist of treating blood specimens with antibody to EpCAM conjugated with magnetic particles, followed by the separation of the cells in a magnetic field. Isolated cells are stained with antibodies against other epithelial markers (cytokeratins 8-18-19 and CD45) in order to distinguish the rare CTC (expressing cytokeratines and lacking CD45 expression) from contaminating white blood cells.[5] There are at least eight different methods to detect CTC.[6,7]

CTC clinical significance is still under investigation, but their potential for early detection of metastases or to predict therapeutic response in patients with advanced disease has been demonstrated for metastatic breast cancer,[8] hormone-refractory prostate cancer[9] and colorectal cancer.[10] The presence of CTC before treatment and a change in their number during treatment itself have a predictive/prognostic value higher than that of the traditional methods. CTC could possibly be used to monitor – not invasively – the acquisition of a *chemo-radio resistant phenotype* in response to chemoradiotherapy and to individualise the treatments (e.g. non small cell lung cancer (NSCLC))[11]; they can be used to estimate the risk of metastases in early stage breast[12] and prostate[13] cancer.

Patients treated for both metastatic and locally advanced head and neck tumours (LAHNC) frequently show recurrence after complete response (CR). The identification of CTC in the blood could ameliorate the prognostic profile already defined by the tumour-node-metastasis (TNM) categories and by other biological prognostic factors (EGFR (epidermal growth factor receptor) or HPV (human papiloma virus) positivity).We therefore started a multi-centric research programme to identify CTC in these patients, using a standardised identification method.

The present paper deals with patients without distant metastases; CTC presence and number and their behaviour after treatment have been studied in the different clinical and pathologic subgroups of our series.

2. Materials and methods

2.1. Characteristics of the patients and treatment

From January 2009 to February 2011, 152 patients with metastatic and non-metastatic head and neck tumours, having signed informed consent, were consecutively submitted to blood sampling (at least 7.5 mL whole blood), at different time intervals before, during and after treatment, to evaluate, using the Cell-Search® technology,[14] the CTC number at diagnosis and its change after the treatment.

Ninety-five patients with non-metastatic, mainly locally advanced disease were included in the study. Twenty-two were excluded from the analysis: 10 for technical problems related to the samples (e.g. coagulation or presence of residual contaminants during CTC detection); twelve because of major deviation from the study procedure after the accrual (treatment discontinuance or early follow-up interruption).

Seventy-three patients were evaluated for the correlation between CTC presence/absence at presentation and other already known prognostic factors (T, N, grade, stage); three of them had surgery after CTC determination.

The correlation between CTC status at diagnosis and response to treatment was studied in the group of 68 patients for whom it was possible to define response to the radical treatment (excluding 3 surgical patients and 2 cases not evaluable for response).

In all patients, blood samples were to be obtained before treatment, after neo-adjuvant chemotherapy and before radiotherapy (when this therapeutic sequence was applied), and at the first follow up visit after the end of the whole primary treatment (usually after a 2–3 months interval since from the closing date of the treatment). Analysis of the correlation between response to treatment and CTC number changes during treatment was possible only in 43/68 patients available for response to treatment. This was due to patient refusal of the second or third blood sampling procedure (10 patients); to change in the Institution responsible for follow up (due to geographical reasons, 4 patients); to technical problems related to the management/transportation of samples (11 patients).

Out of 73 patients, 53.4%, 13.7% and 13.7% had respectively oropharynx, larynx and nasopharynx cancer; all of them, apart the sinonasal undifferentiated carcinoma (SNUC) cases, had squamous cell carcinomas; 45.2% of them had G3-4 disease; 39.7% had T4 disease; 64.6% had N2 nodal involvement. Seventy-six percent of the patients had stage IV disease [TNM 7th edition]. Performance status according to the Karnofsky Scale was $\geqslant 70$ in most patients (91.7%).

Diagnostic work-up included different combinations of computerised tomography (CT, 68%), magnetic resonance imaging (MRI, 61.6%), neck sonography (US, 53.4%), positron-emission tomography, PET (9.6%).

Forty-two percent of the patients were treated with radiotherapy concomitant to chemotherapy or Cetuximab, 34% had (additionally) neo-adjuvant chemotherapy (CHT) and only a minority (19%) was given exclusive radiotherapy (RT) (Table 1).

Three *treatment categories* were identified: RT alone, RT after neoadjuvant CHT – with or without concomitant systemic treatment –, and RT concomitant with systemic treatment (Cisplatinum based chemotherapy or Cetuximab).

A dose of 70–69 Gy (2–2.3 Gy/fraction) was given to the tumour and clinically positive lymph-nodes, with 3D conformal radiotherapy (3DCRT) or intensity modulated radiation therapy (IMRT). Clinically negative nodal areas at risk received a dose of 50–54 Gy (2–1.8 Gy/fraction). The same doses and techniques were used in patients treated also with CHT.

When CHT was given before RT it consisted of 2 or 3 cycles of the TPF schema (docetaxel 75 mg/m^2, cis diamine dichloro platinum (CDDP) 75 mg/m^2 and 5-fluoruracil 750 mg/m^2, intravenous (i.v.), every three week). Chemotherapy concomitant to RT consisted of weekly CDDP (40 mg/ m^2 i.v.). Cetuximab was given at standard doses (400 mg/m^2 i.v. infusion one week before RT and then 200 mg/m^2 weekly, concomitant with RT).

Response to treatment was evaluated with the same imaging techniques used at diagnosis (CT, MRI, US and PET). This was done after chemotherapy, in case of neo-adjuvant treatment, and 2–3 months after treatment completion, in all the patients. Response was

Table 1
Clinical and therapeutic features of the entire series.

	Number	%
Sex		
M	56	76.7
F	17	23.3
IK[b]		
90–100	32	43.8
70–80	35	47.9
$\leqslant$60	6	8.3
Site		
Nasopharynx	10	13.7
Oropharynx	39	53.4
Oral cavity	3	4.2
Hypofarinx	5	6.8
Larynx	10	13.7
Paranasal sinuses	6	8.2
SCC		
Grade 1	1	1.4
Grade 2	20	27.4
Grade 3	21	28.8
Grade 4[c]	9	12.3
SNUC[d]	3	4.1
Not known	19	26
T class		
1	7	9.6
2	19	26
3	18	24.7
4	29	39.7
N class		
0	18	24.4
1	8	11
2	47	64.6
3	0	0
Stage		
I	1	1.4
II	5	6.8
III	11	15.1
IV	56	76.7
Treatment		
RT alone	14	19.2
RT + concomitant CHT/cetuximab	31	42.5
CHT neo + RT +/− CHT/cetuximab	25	34.2
Surgery as part of the treatment[a]	3	4.1

[a] Circulating Tumour Cells (CTC) blood sample was obtained before surgery.
[b] IK = Karnofski Index.
[c] Some pathologists defined as SCC Grade 4 the most undifferentiated cases, using a 1–4 scale instead of a 1–3 one.
[d] SNUC, sinonasal undifferentiated carcinoma; RT, radiotherapy; CHT, chemotherapy; SCC, squamous cell carcinoma.

classified according to the Response Evaluation Criteria in Solid Tumours (RECIST) criteria.[16,17] Subsequent follow-up included bimonthly clinical examination and diagnostic imaging every 4 months during the first year; thereafter, follow up intervals became progressively longer. Median follow up was equal to 409 days.

2.2. CellSearch® technology and other laboratory techniques

CellSearch® Epithelial Cell Kit/CellSpotterTM Analyzer® technology isolate CTC from whole blood by immunomagnetic enrichment using ferrofluids coated with EpCam specific antibodies. Subsequently, the isolated cells were stained with fluorescent monoclonal antibodies for epithelial cells (cytokeratins 8, 18, 19), leucocytes (CD45) and a nuclear staining dye, and thereafter enumerated using a semi-automated fluorescence microscope. CTC were defined as nuclear cells, expressing cytokeratins and lacking CD45 expression.[6] CellSearch® technology has been already approved by the Food and Drugs Administration (FDA) for clinical use in breast and colon cancer patients and – as very recently demonstrated – cells from head and neck squamous cell cancer could be identified in peripheral blood using this method.[15] Before starting the clinical application of the method, the system was tested in preclinical models by C.A., S.G., R.V.

CTC determination was performed in a control group of nine healthy volunteers, after informed consent. CTC determination was considered positive if at least one cell, with the defined characteristics, was identified. The total number of CTC identified was registered.

Changes in the number of CTC during the treatment were categorised as 'neg–neg' if no CTC were present at diagnosis and at the end of the treatment; 'neg–pos' if no CTC were present at diagnosis and were instead detected at the end of treatment; 'pos-less' if the number of CTC decreased during the treatment; 'pos-more' if the number of CTC grew during the treatment; 'pos–neg' if CTC disappeared after treatment.

2.3. Statistical analysis

The statistical correlation between the different variables was evaluated with the Fisher exact probability test or with χ^2-test, using SPSS 17th edition®. Correlations were considered statistically significant when the p value was less than 0.05. Throughout the text and in all the Tables, p value has been reported only when corresponding to a statistically significant difference.

3. Results

No CTC were identified in nine healthy subjects tested (100%).

Table 2
Circulating Tumour Cells (CTC) positivity before treatment in relation with different clinical features.

	CTC+	CTC −	CTC +/Tot (%)	p (χ2)
Site				
Nasopharynx	0	10	0/10 (−)	0.05
Oropharynx	5	34	5/39 (13%)	
Oral cavity	0	3	0/3 (−)	
Hypofarinx	2	3	2/5 (40%)	
Larynx	1	9	1/10 (10%)	
Paranasal sinuses[a]	3	3	3/6 (50%)	
Grade				
1–2	4	17	4/21 (19%)	NS
3–4	5	28	5/33 (15%)	
Not known	2	17	2/19 (10%)	
T class				
1	0	7	0/7 (0)	NS
2–4	11	55	11/66 (17%)	
N class				
0–1	4	22	4/26 (15%)	NS
2	7	40	7/47 (15%)	
Stage				
I–II–III	1	16	1/17 (6%)	NS
IV	10	46	10/56 (18%)	
T + N categorisation				NS
T1 N0–1-2 T2 N0–1 T3 N0	1/16 (6.3%)			
T2 N2 T3 N1–2 T4 N0–1-2	10/57 (17.5%)			

χ^2-test $p = 0.05$, for the differences in CTC positivity according to the site of origin of the tumour; NS = not significant.

[a] 2 of the 3 CTC positive cases were sinonasal undifferentiated carcinoma (SNUC).

CTC were identified at diagnosis in 11/73 patients with locally advanced disease (15.1%), more frequently in patients with oropharyngeal (5/39, 13%), hypopharyngeal (2/5, 40%) and paranasal sinuses cancer (3/6, 50%). No CTC were identified in the 13 patients with oral cavity and nasopharyngeal cancer ($p = 0.05$; Table 2).

Median CTC number was 2 (range 1–43). Forty-three and 10 CTC were identified in two patients with locally advanced SNUC.

In 4/73 (5.5%) patients CTC became apparent only during the treatment.

3.1. Correlation with clinical prognostic factors

CTC were identified in 18% (10/56) of the patients with stage IV disease versus 6% (1/17) of those staged as I–II–III; CTC were absent in the 7 patients with T1 disease while they were detected in 17% (11/66) of those with T 2–4 disease. CTC positivity at diagnosis

Table 3
Circulating Tumour Cells (CTC) positivity in relation to T and N categories in the entire series (the two groups with low and high tumour burden are identified as in Table 2).

N	0	1	2	Tot
T1	0/1	0/1	0/5	0/7
T2	1/4	0/1	3/14	4/19
T3	0/4	0/3	1/11	1/18
T4	3/9	0/3	3/17	6/29
Tot	4/18	0/8	7/47	11/73

according *also* to nodal status was more frequent in patients with more advanced nodal *and* primary tumour extent – i.e. with larger tumor burden (Tables 2 and 3). All these analyses were repeated excluding the patients with nasopharynx and oral cavity cancer, with the same results (not shown).

3.2. Correlation with response to the treatment

In 68 patients eligible for clinical response analysis, 43/68 (63%) had CR, 20/68 (30%) had PR (partial response) and 5/68 (7%) had NC (no change).

A correlation between CTC positivity at diagnosis and clinical response might be suggested, even if not statistically significant, because the proportion of patients with CTC positivity was much lower in those obtaining a CR than in those obtaining a PR or no response (4/43, 9.3% versus 6/25, 24%).

In 43 patients with multiple blood samples, the correlation between the changing number of CTC during treatment and clinical response was strong. Ninety percent (37/41) of those obtaining a clinical response (complete or partial) did not show CTC at diagnosis or during treatment or became negative, as opposed to none of the non-responding patients ($p = 0.017$; Table 4).

3.3. Correlation with relapse patterns and survival

We tried to relate relapse pattern and survival with CTC presence in 68 patients with evaluable response. Pattern of relapse (according to response to treatment) was shown in Table 5. After a median follow-up of 409 days 57 patients were alive (54% of them without evidence of disease); nine died with disease and two were lost to follow-up.

Table 4
Correlation between clinical response and Circulating Tumour Cells (CTC) number changes during the treatment (43 patients).

	CR (complete response)/ PR (partial response)	NC (no change)
'neg–neg' 'pos–neg'	37 (90%)	0
'neg–pos' 'pos–less' 'pos–more'	4 (10%)	2 (100%)
Tot	41	2

Table 5
Evolution of the clinical findings during follow up according to treatment response (68 patients).

Evolution during follow-up	Response to treatment	
	CR	PR/NC
CR maintained	35	-
SD	-	8
Local relapse/progression	3	9
Distant relapse	4	7
Not evaluable	1	1
Tot	43	25

CR: complete response; PR: partial response; NC: no change; SD stable disease.

No significant correlation was evidenced between CTC positivity at diagnosis and survival or relapse.

However, when the analysis was limited to the 43 patients submitted to multiple samples, a correlation was evident (even if not statistically significant) between the change in the number of CTC and the frequency of recurrence or progression or metastatic disease. Only 3/31 patients (9,5%) who remained in CR or did not show progressive disease (after less than CR to treatment) were CTC positive at the end of the treatment; in 30% of those who relapsed or showed progressive disease, instead, CTC were identified at the end of the treatment.

Finally, considering vital status at the last follow-up, the proportion of patients with CTC identified at the end of the treatment was much lower (1/21, 5%) among those alive with no evidence of disease than in those alive (2/17, 12%) or dead (3/5, 60%) with disease ($p = 0.009$; Table 6).

4. Discussion

Patients with LAHNC, after CR to primary treatment, showed often early local or distant recurrence,[18] that could be the consequence of 'dormant' microscopic disease persistent in the primary tumour site or in distant sites.[19]

The search for prognostic and predictive molecular and biological factors is very active.[20–24]

Microscopic disease in bone marrow and 'central venous blood' has been searched attempting to predict recurrence and metastatic disease in patients with operable head and neck cancer.[23] The value of these invasive methods has to be confirmed.

CTC had been firstly identified in the peripheral blood of surgically treated head and neck cancer patients, using an immunocytochemistry assay with established monoclonal antibodies, combined with an enrichment system with anti-human epithelial antigen

Table 6
Correlation between Circulating Tumour Cells (CTC) modification during the treatment and survival (43 patients).

	Alive NED[b]	Alive with disease	Dead with disease
'neg-neg' 'pos-neg'	20 (95%)[a]	15 (88%)	2 (40%)
'neg-pos' 'pos-less' 'pos-more'	1 (5%)	2 (12%)	3 (60%)
Tot	21	17	5

[a] Fisher exact test $p = 0.009$.
[b] NED = No Evidence of Disease.

(EpCAM). However, the number of patients was too small to show clinically meaningful prognostic correlations and to define CTC role in the clinical management of these patients.[25]

More recently, Hristozova identified CTC using flow cytometric analysis of CD45 epithelial cell adhesion molecule + cytokeratin + cells, validated by nested RT-PCR (reverse transcription-polymerase chain reaction) analysis, reporting a statistically significant correlation between CTC positivity and the presence of lymph node metastases (N0-2a versus N2b-N3), in unresectable LAHNC patients[26]; the correlation between response to the treatment and CTC presence was not analysed.

Jatana et al. developed a method based only on negative depletion of normal cells,[27,28] trying unsuccessfully to correlate CTC positivity with the already known prognostic factors, but showing a statistically significant difference in disease free survival favoring patients without CTC at diagnosis.

None of the reported data used the same repeatable method to identify CTC in the blood.

The advantage of the Cell-Search System®, even considering the bias related to the 'positive selection technique', is its reproducibility. A very recent paper documented CTC positivity, using this method, in a little group of 15 patients with locally advanced and metastatic head and neck cancer.[15] To date, no information exists about the identification of CTC in head and neck cancer, using this method in a homogenous group of patients; our series includes different tumour sites and histology, even if is the largest reported.

Our data confirm partly what already known from others studies. An high frequency of CTC positivity has been recorded in oropharynx cancer patients (13%) and an even higher frequency in the small number of cases with hypopharyngeal (40%) and paranasal sinuses neoplasms (50%) suggesting both a higher expression of epithelial markers in this kind of disease and a higher probability of systemic spread. On the contrary, all the patients with oral cavity ($n = 3$) and nasopharyngeal cancer ($n = 10$) were negative for CTC detection. These data should, however, be interpreted cautiously, due to the limited number of patients in each subgroup.

Differently from Hristozova but in accordance with Jatana et al.,[26,27] we found a correlation with some clinical and prognostic factors. In patients with stage IV disease and with high 'tumour burden' (Table 3), CTC positivity was more frequent than in those with stages I–II–III (18% and 6%, respectively). The histological grade of disease did not seem related to CTC positivity, possibly because of the limited number of cases in each category. SNUC seems to be characterised by the presence of a very high number of CTC in the peripheral blood (and 2/3 paranasal sinus cancer cases with CTC positivity had this histology, in agreement with the well known frequency of nodal/distant metastases in these patients).

Data relating the reduction of the number of CTC during treatment with better clinical response and survival had been reported for metastatic breast, colon and prostate cancer.[4–6,25] The influence of CTC detection on survival is not univocal and has not been confirmed in non-metastatic breast[12,29–31] and prostate cancer.[13]

No information regarding the relationship between clinical response to non-surgical treatment and the change of the number of CTC *during* the treatment itself is available for patients with head and neck cancer. Our data demonstrate a statistically significant ($p = 0.017$) correlation between partial or CR and absence or disappearance of CTC during the treatment (Table 4). CTC negativity is related with non-progressing disease after both CR and PR; these findings could support the available evidence, reported by Jatana et al., of a better disease free survival in patients without CTC at diagnosis.[27] However, we did not attempt to search a relationship between CTC positivity and actuarial disease free survival in our series, because of the short median follow-up. Moreover, the meaning of a neg/neg and of a pos/neg sequence may not be the same; unfortunately, the small number of cases prevented us from a more detailed analysis and may suggest increasing the number of patients studied.

Similarly, the origin of CTC in blood samples taken during/after treatment in patients who were initially 'CTC negative' could be ascribed to the evolution of distant sites of disease, or to the selection of resistant tumour cell clones in the primary tumour, but only a larger series could shed some light on this issue.

5. Conclusions

With the limit of the relatively small number of cases studied and of the short follow-up period, these preliminary data show a possible role for CTC determination in head and neck cancer. CTC presence seems related to tumour burden; no correlation was evident between the CTC positivity at diagnosis and response to non-surgical treatment. The most important variable seems to

be the change of CTC number during treatment: better response and better survival were evident if CTC were always absent or if they disappear during the treatment. Further analysis is needed on a larger number of cases and after a longer follow up, to confirm or reject this hypothesis.

Conflict of interest statement

All authors declare that they do not have any financial and personal relationship with other people or organisations that could inappropriately influence their work.

Role of the funding source

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